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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

009476

MAY 4 1992

OFFICE OF
PESTICIDES AND TOXIC
SUBSTANCES

MEMORANDUM

SUBJECT: DowElanco Response to Previous EPA Reviews of Mutagenicity Studies on 2,4-D TIPA, 2,4-D IPA, and 2,4-D BEE

TO: Coombs/Waldrop, PM 71
SRRD (H7508W)

FROM: Byron T. Backus, Ph.D., Toxicologist Byron T. Backus 4/29/92
Toxicology Branch 2
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THROUGH: K. Clark Swentzel K. Clark Swentzel 4/29/92
Section Head, Review Section 2
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and

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HED (H7509C) Marcia van Gemert 4/30/92

DP Barcode D162581 Submission: S406334

Project No. 2-0393 ID#: 030025

Tox. Chem. 030035 (2,4-D TIPA, Caswell #315AE), 030025 (2,4-D IPA, Caswell #315U), and 030053 (2,4-D BEE, Caswell #315AI)

Study Classification Revisions: The 2,4-D TIPA mouse micronucleus assay is reclassified as acceptable (original study in MRID 414783-02, with additional information in MRID 420157-02). This study satisfies guideline data requirements for a micronucleus assay (84-2(3)(B). The Ames assays for 2,4-D TIPA (MRID 413882-02 and 417979-01, with additional information in 420157-01), for 2,4-D IPA (MRID 413882-03 and 417979-02, with additional information in 420157-01), and 2,4-D BEE (MRID 413882-04 and 417979-03, with additional information in 420157-01) are reclassified as conditionally acceptable, and would satisfy the guideline data requirements for a Salmonella typhimurium assay (84-2(1)), provided

1 of 29

the registrant conducts two studies on both 2,4-D BEE and 2,4-D IPA, and one study on 2,4-D TIPA, to meet the new (March, 1991) mutagenicity guidelines, Series 84 of Subdivision F, Addendum 9 to the Pesticide Assessment Guidelines (see attached copy). Specifically, these studies would include a mammalian cells in culture forward gene mutation assay (such as a study utilizing mouse lymphoma L5178Y cells and the thymidine kinase gene locus, maximizing assay conditions for small colony expression and detection) for all 3 compounds, and an in vivo cytogenetics study (preferably using rodent bone marrow) using either metaphase analysis for chromosomal aberrations or a micronucleus assay (for 2,4-D IPA and 2,4-D BEE). Note that a UDS assay would not be appropriate.

Action Requested:

Review the registrant's responses to a number of mutagenicity study reviews on 2,4-D BEE, 2,4-D TIPA, and 2,4-D IPA. Six of the studies (the UDS and mouse micronucleus assays) were reviewed at Dynamac, while the Salmonella (Ames) studies were reviewed within HED.

Comments and Recommendations:

1. In the UDS studies, each of the original EPA (Dynamac) reviews included the comment: "The excessively high background cytoplasmic and nuclear grain counts in all groups make it difficult to distinguish between a positive and negative genotoxic response." The average cytoplasmic grain counts ranged from 34.1 to 38.0 in the solvent controls, and from 31.0 to 50.5 for the positive controls. These cytoplasmic grain counts are excessive, even taking into consideration that "the cytoplasmic grain count for each cell was estimated from one nuclear-sized area in the cytoplasm that was judged to have the highest number of silver grains." Also, the reporting of accidental exposure to light with some coverslips (reported as not being subsequently evaluated) raises our concerns that the coverslips that were evaluated may have also been accidentally exposed to light.

   While there was no evidence for any of the three compounds of an increase in mean net nuclear grain counts at any dose level, with the heavy nuclear labeling in the positive controls it is difficult to see how cells in S-phase could be differentiated from those in which UDS was occurring. While it is evident that 2-AAF at 2.223 µg/mL (=0.01 mM) elicited a positive response, it is possible that a somewhat weaker positive control (perhaps 2-AAF at a lower concentration such as 0.001 mM would have given considerably more equivocal results. For these reasons, these studies remain classified as unacceptable.

2. All of the in vivo mouse bone marrow micronucleus tests were previously classified as unacceptable because there was insufficient evidence that the highest doses tested were adequate. After examining the reviews, this reviewer concurs with the original classifications of the 2,4-D BEE and 2,4-D IPA studies as unacceptable. In the 2,4-D BEE assay, there were no mortalities among the 15 males and/or 15 females at the HDT (375 mg/kg). For 2,4-D IPA there were no mortalities at the HDT (750 mg/kg) of the assay. The lack of mortalities in these studies (and no reporting of any adverse signs) strongly suggests higher doses could have been administered (more than 15 animals/sex could have received the HDT so that replacement animals would have been available). It is noted that a shift in the PCE to NCE ratio may be a reflection of the biological response of the target (bone marrow) tissue, and the Agency takes this into consideration in determining whether the HDT was adequate.

   For 2,4-D TIPA, 1/15 males and 1/15 females died at the HDT (750 mg/kg). While symptoms were not reported, it is concluded that the occurrence of mortality in 2 animals is sufficient to demonstrate that the HDT was adequate. The classification of the 2,4-D TIPA mouse micronucleus assay is then upgraded to acceptable.

3. After reevaluation of the Ames studies (and taking into account the registrant's comments regarding the spontaneous incidence of TA98 and TA100 revertants on Oxoid L-11 agar) it is concluded that the Ames studies and their negative findings can be conditionally accepted, and do not need to be repeated, provided the registrant conducts two studies on 2,4-D BEE and 2,4-D IPA, and one study on 2,4-D TIPA (since the mouse micronucleus has been reclassified as acceptable) to meet the new (March, 1991) mutagenicity guidelines, Series 84 of Subdivision F, Addendum 9 to the Pesticide Assessment Guidelines (see attached copy). Specifically, these studies would include a mammalian cells in culture forward gene mutation assay (such as a study utilizing mouse lymphoma L5178Y cells and the thymidine kinase gene locus, maximizing assay conditions for small colony expression and detection) for all three compounds, and an in vivo cytogenetics study (preferably using rodent bone marrow) using either metaphase analysis for chromosomal aberrations or a micronucleus assay for 2,4-D BEE and 2,4-D IPA. Note that UDS assays are not included.

Discussion:

Regarding the UDS studies (original submissions in MRID nos. 414981-01, 414981-02, and 414981-03), each of the reviews had the same following comment: "Although the dose range tested and the protocol designed for the study appeared to be adequate, technical problems with the assay preclude an accurate assessment of the results. The excessively high background cytoplasmic and nuclear grain counts in all groups make it difficult to distinguish between a positive and negative genotoxic response." In addition, as noted in the most recent response from the registrant, there was only one acceptable slide/treatment/assay since, "during the coating of the slides with photographic emulsion, the slides were accidentally exposed to a pulse burst of light."

The following "representative" (solvent and positive controls, two highest doses evaluated from each of two duplicate assays) results are provided in the Dynamac reviews:

2,4-D TIPA:

Treatment	Dose/mL	Cells Scored	Average Cytoplasmic Grain Count	Mean Net Nuclear Grain Count ± S.D.
Solvent control				
Culture medium	--	100	35.5	-12.4 ± 6.4
	--	100	36.5	-11.9 ± 6.4
Positive control				
2-AAF	2.223 µg	50	50.5	49.5 ± 7.3
	2.223 µg	50	45.1	55.8 ± 8.8
2,4-D TIPA	500.0 µg	100	29.4	-9.2 ± 5.4
	500.0 µg	100	36.9	-10.8 ± 6.3
	166.7 µg	100	40.5	-15.3 ± 7.4
	166.7 µg	100	42.0	-10.9 ± 6.7

2,4-D IPA:

Treatment	Dose/mL	Cells Scored	Average Cytoplasmic Grain Count	Mean Net Nuclear Grain Count ± S.D.
Solvent control				
Culture medium	--	100	37.6	-13.0 ± 6.6
	--	100	38.0	-14.5 ± 7.2
Positive control				
2-AAF	2.223 µg	50	50.5	49.5 ± 7.3
	2.223 µg	50	45.1	55.8 ± 8.8
2,4-D IPA	500.0 µg	100	35.5	-11.6 ± 7.2
	500.0 µg	100	33.7	-11.7 ± 6.7
	166.7 µg	100	41.2	-15.1 ± 7.4
	166.7 µg	100	42.5	-16.3 ± 7.5

2,4-D BEE:

Treatment	Dose/mL	Cells Scored	Average Cytoplasmic Grain Count	Mean Net Nuclear Grain Count ± S.D.
Solvent control				
DMSO	10 µL	100	34.1	-16.6 ± 7.1
	10 µL	100	34.1	-13.0 ± 6.2
Positive control				
2-AAF	2.223 µg	50	35.6	64.4 ± 8.0
	2.223 µg	50	31.0	69.0 ± 7.3
2,4-D BEE	500.0 µg	100	36.2	-14.6 ± 7.8
	500.0 µg	100	31.5	-14.4 ± 7.0
	166.7 µg	100	34.5	-15.1 ± 6.9
	166.7 µg	100	33.6	-13.9 ± 6.5

On examination of the data above and comments from the registrant, the following points can be made:

1. The sum of (average cytoplasmic grain count) + (mean net nuclear grain count) for the positive controls is equal to 100 or 99.9, which indicates that all (or almost all) of the nuclei evaluated from this group were scored as having 100 grains (also, positive control values for the 2,4-D TIPA and 2,4-D IPA studies are the same, suggesting that the UDS assays for these two actives used the same positive control cultures). This is consistent with the registrant's response to previous reviews in which it is stated (for positive controls): "The nuclear labelling was so heavy that an accurate counting of individual nuclear grains was not possible and a minimum grain count of 100/nucleus was only an estimate."

2. The average cytoplasmic grain counts are excessive, even taking into consideration that "the cytoplasmic grain count for each cell was estimated from one nuclear-sized area in the cytoplasm that was judged to have the highest number of silver grains." In the first assay with the 2-AAF positive control for 2,4-D TIPA and 2,4-D IPA, slightly more than half (50.5%) of the reported value for nuclear grains was from the background, and the value (45.1) was nearly as high in the second assay. While it is evident that 2-AAF at 2.223 µg/mL (= 0.01 mM) elicited a positive response, one of this reviewer's concerns is that a somewhat weaker positive control (perhaps 2-AAF at a lower concentration such as 0.001 mM) would have given considerably more equivocal results.

3. With the heavy nuclear labeling in the positive control cells, it is difficult to see how cells in S-phase could be differentiated from those in which some UDS was occurring. If some heavily labelled cells were present on slides from cultures

exposed to the test materials, they might simply be classified as cells in S-phase

In the material that the registrant has submitted from the open literature (Tong, McQueen, Ved Brat and Williams, 1988, and McQueen, Rosado and Williams, 1989), average cytoplasmic grain counts are not reported. However, it is noteworthy that the for the positive control (in the study by Tong et al.) 2-aminofluorene at 0.1 mM the mean net grains/nucleus was 72.2; despite this relatively high value there is no indication that there was any necessity for assigning grain count values of 100 to nuclei (and the text indicates that counts were made using an Artek electronic counter). Further, the sensitivity of the assay was demonstrated by testing 2-aminofluorene at 0.01 mM.

For these reasons, these studies remain classified as unacceptable.

All of the in vivo mouse bone marrow micronucleus tests were classified as unacceptable because there was insufficient evidence that the highest dose was a MTD.

In the assay conducted on 2,4-D BEE, the mortalities in the preliminary dose-ranging test were as follows:

Dose (mg/kg)	Mortalities/Dosed
250	0/10
500	8/10
1000	10/10
2000	10/10

On the basis of these findings, 375 mg/kg was selected as the highest dose level in this study (the lower dose levels were 37.5 and 125 mg/kg).

Within the study itself, there were no mortalities among the 15 males and/or 15 females which were dosed at 375 mg/kg. Further, there was no indication within the report that any signs of toxicity occurred at this dose level (signs may or may not have been present, as the text in MRID 420157-04 states: "Clinical signs of toxicity were not evaluated either in the initial range-finding study or in the micronucleus test, and hence data on overt toxicity in the treated animals was not reported."). However, as was noted in the original review: "without evidence of compound toxicity or interaction with bone marrow cells, there is no assurance that the maximum tolerated dose (MTD) was achieved."

We concur with the conclusions of the original review then that: "The study is unacceptable and should be repeated with a level of 2,4-D BEE that clearly demonstrates that the MTD was achieved." In order to demonstrate that the highest dose is adequate there could either be some mortalities related to exposure to the test substance (the number of animals dosed at this level would then have to be in excess of 15/sex, so that replacement animals would be available), or frank symptoms of compound toxicity (either to the animals or bone marrow cells) would have to be present and reported.

In the mouse micronucleus assay conducted with 2,4-D TIPA, the mortalities in the preliminary dose-range test were as follows:

Dose (mg/kg)	Mortalities/Dosed
500	0/10
1000	2/10
2000	8/10

In the original review it is stated that the 2 animals which died at 1000 mg/kg were both females, and the deaths occurred at least 4 days after treatment.

The dose levels tested in the subsequent micronucleus assay were 75, 250, and 750 mg/kg. At 750 mg/kg, 1/15 males and 1/15 females died within 72 hours of dosing. While symptoms were not reported, it is concluded that the occurrence of mortality in 2 animals is sufficient to demonstrate that the HDT was adequate. The classification of the 2,4-D TIPA mouse micronucleus assay is then upgraded to acceptable.

In the mouse micronucleus assay with 2,4-D IPA, the mortalities in the preliminary dose-range test were as follows:

Dose (mg/kg)	Mortalities/Dosed
500	0/10
1000	2/10
2000	10/10

As with 2,4-D TIPA, doses administered in the micronucleus assay were 75, 250, and 750 mg/kg. There were no mortalities at any of these doses of 2,4-D IPA.

The registrant has cited the paper by Mavournin et al. (1990), from the U.S. Environmental Protection Agency Gene-Tox Program. This paper recommends ≈50% of the LD_{50} as the highest dose in this assay. However, with respect to testing pesticides, we do not accept this recommendation. In short, this study remains classified as unacceptable.

Regarding the Ames assays, the major question regarding the acceptability of these studies is the lower-than-usual revertant incidence observed for TA98 and TA100. According to the most recent response (MRID 420157-01) from the registrant: "In the studies submitted, the spontaneous reversion frequency ranged from 6 to 22 for TA98 and from 38 to 77 for TA100. The reviewer, citing an acceptable range of 15-50 for TA98 and 100-200 for TA100, concluded that the genetic characteristics of the tester strains used in our studies were compromised. In the response submitted earlier, it was stated that the some what lower reversion frequencies in our studies was due to the use of Oxoid L-11 agar in the preparation of minimal medium petri plates and not due to the loss of any of the genetic characteristics of the strains."

After examining the studies and initial responses from the registrant (in MRIDs 413882-02, -03, -04, 417979-01, -02, and -03) the following comments appear to be appropriate:

1. The R-factor plasmid, pKM101, is normally associated with ampicillin resistance. There is nothing within the reports (or responses) that specifically states that the TA98 and/or TA100 strains were tested for ampicillin resistance; the closest it gets to such a comment is (see, for example, p. 6 of 417979-01): "All of our overnight cultures for the mutagenicity assays were started from tester strains stored as frozen permanents at -100°C or below. For each inoculation, a new vial of the frozen permanent was used. The genetic characteristics of the strains were verified prior to freezing and the frozen stocks were used within a year of storage."

2. There is nothing within the original reports as to what the laboratory considers as acceptable revertant ranges for the different tester strains. It is noted, however, that in the 2,4-D BEE assay the solvent control for TA98 in the first assay yielded a mean of 22 revertants/plate; in the second assay there was a "mean" of 6 revertants/plate (this was the value for only one plate, as two plates were "contaminated with fungus"), and the assay for this strain was then repeated a third time (mean no. of revertants in solvent control: 11). Presumably (although the report does not say so) the third assay was done because the results from the second were in some way unsatisfactory (incidence of revertants outside the acceptable range for solvent controls in the absence of S9?).

3. It is possible to compare results for TA100 and TA1535 (the strain from which it originated); in both cases the positive control in the absence of S9 was sodium azide (25 µg/plate) and the positive control in the presence of S9 was 2-anthramine (3 µg/plate). The positive control in the absence of S9 elicited

essentially the same response in both strains; otherwise (including positive controls in the presence of S9, solvent controls both with and without S9) there was generally an approximately 10X incidence in revertants in the TA100 as compared with TA1535. While this observation has to be interpreted cautiously (there is no guarantee that the TA100 and TA1535 strains in this study were genetically identical except for the presence of the plasmid in the TA100), the findings are at least consistent with this TA100 being more susceptible to the appropriate mutagens than TA1535.

4. After taking the above observations into consideration (as well as the registrant's comments regarding Oxoid L-11 agar) it is concluded that the Ames studies and their negative findings can be conditionally accepted, and do not need to be repeated, provided the registrant conducts two studies on both 2,4-D BEE and 2,4-D IPA, and one study on 2,4-D TIPA, to meet the new (March, 1991) mutagenicity guidelines, Series 84 of Subdivision F, Addendum 9 to the Pesticide Assessment Guidelines (see attached copy). Specifically, these studies would include a mammalian cells in culture forward gene mutation assay (such as a study utilizing mouse lymphoma L5178Y cells and the thymidine kinase gene locus, maximizing assay conditions for small colony expression and detection) for all 3 compounds, and an in vivo cytogenetics study (preferably using rodent bone marrow) using either metaphase analysis for chromosomal aberrations or a micronucleus assay (for 2,4-D IPA and 2,4-D BEE). Note that a UDS assay would not be appropriate.

TOXCHEM NO. 030035- Dichlorophenoxyacetic acid, triisopropanolamine salt FILE LAST PRINTED: 04/29/92

CITATION	MATERIAL	ACCESSION/ MRID NO.	RESULTS	TOX CAT	CORE/GUIDE/ DOCUMENT#
83-3(a) Developmental Toxicity Study Species: rat Bio/dynamics Inc. 89-3464; 89-346A; 4/10/90	2,4-D TIPA salt tech. 72.3%; Lot No. AGR 276428	415271-05	RANGE FINDING STUDY: Levels tested in CD (Sprague-Dawley derived) rats by gavage on days 6-15 of gestation. In 89-3464 at: 0, 95, 190 & 280 mg/kg; 89-3464A at 0, 280, 375 & 465 mg/kg. Maternal NOEL = 190 mg/kg. Maternal LOEL = 280 mg/kg. Develop. Tox. NOEL & LOEL was not assessed. Doses: 0, 32.5, 100 and 325 mg/kg.		Supplementary 008186
83-3(a) Developmental Toxicity Study Species: rat Bio/dynamics Inc. 89-3463; 5/25/90	2,4-D TIPA salt tech. 72.2%; lot No. AGR 276428	415271-02	Levels testedin CD (Sprague-Dawley derived) rats by gavage at: 0, 32.5, 100 and 325 mg/kg/day on days 6-15 of gestation. Maternal NOEL = not achieved. Maternal LOEL = 32.5 mg/kg. Develop Tox. NOEL = 32.5 mg/kg. Develop. Tox. LOEL = 100 mg/kg (skeletal malformations/variations).		Minimum 008186
82-1(a) Feeding-3 month Species: rat Dow Chemical Co. K-008866-006; 09/04/91	2,4-D - TIPA salt 72.2%; acid equivalent 37.7%	420214-02	Male and female F-344 rats were fed diets containing 0, 2, 28, 187 or 560 mg/kg/day for 13 weeks. No treatment-related effects at 2 or 28 mg/kg /day. Treatment-induced effects at 187 mg/kg were decreases in mean body weight gain, minor alterations in hematology, clinical chemistry, & urinalysis, changes in organ weights, and histopathological changes in the kidneys, liver and adrenal glands. Treatment-related effects at 560 mg/kg/day included decreases in body weight gain and food consumption, alterations in hematology, clinical chemistry & urinalysis, changes in organ weights, gross pathology and histopathological changes in the eyes, liver, kidneys, and thyroid. NOEL = 28 mg/kg/day. LOEL = 187 mg/kg/day based on decreases in body weight, alterations in hematology, clinical chemistry & urinalysis, increases in relative kidney weight & histopathological lesions in the kidneys, liver and adrenal glands.		Guideline 089466
82-2 Dermal-3 week Species: rabbit Dow Chemical Co. K-008866-004; 12/8/89	2,4-D Triisopropylamine salt 72.2%	414079-02	Dose levels tested: 0, 100, 350 and 1000 mg/kg/day. A few animals at each level showed very slight to slight inflammatory reactions confined to the superficial dermis only; no epidermal lesions were seen. No evidence of systemic toxicity. NOEL = 1000 mg/kg/day (HDT) for dermal & systemic toxicity.		Minimum 008185
84-2(a) Mutagenic-Ames Species: salmonella Dow Chemical, Texas TXT;K-00866-007; 12/12/89	2,4-D triisopropanolamine (2,4-D TIPA)	413882-02 417779-01 420157-01	This study does not satisfy guideline 84-2(a) requirements for mutagenicity testing. A final conclusion can not be reached due to the following reported deficiencies. (1) The spontaneous revertants for the R-factor strain (TA98, TA100) reported in this study are considerably lower than the normal range of revertants for these strains recommended for performing the Ames test. (2) The lack of information as to the test material stability and supporting analytical data to confirm actual test materials concentrations used in the assays. The study may be upgraded upon resolution of reported deficiencies. ~~The additional information/explanation unacceptable. Study cannot be upgraded and should be repeated.~~ Evidence given (MRID 420157-01) that number of histidine revertants in TA98 & TA100 is less in this assay		Unacceptable 087976 Conditionally Acceptable xxxxxx

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TOXCHEM NO. 030035- Dichlorophenoxyacetic acid, triisopropanolamine salt FILE LAST PRINTED: 04/29/92

CITATION	MATERIAL	ACCESSION/ MRID NO.	RESULTS	TOX CAT	CORESHADE/ DOCUMENT#
84-4 Mutagenic-unscheduled DNA synt Species: rat hepatocytes Dow Chem; Lake Jackson Res. TXT:K-000866008; 5/3/90	2,4-D TIPA 72.2%	414981-02	Dose levels tested: 5-500 ug/mL. Technical problems; excessively high background cytoplasmic & nuclear grain counts in all groups.		Unacceptable 008572 Unacceptable xxxxxx
84-4 Mutagenic-micronucleus assay Species: mice bone marrow Dow Chem; Lake Jackson Res. TXT:K-000866009; 4/24/90	2,4-D TIPA 72.2%	414783-02	Dose levels tested: 75, 250, or 750 mg/kg-oral. MTD not used; no evidence of toxicity or interaction with target tissue (bone marrow cell) Since 1/5 males and 1/5 females died at the HDT (750 mg/kg), this is considered sufficient evidence that this was an adequate dose. No evidence was observed of an increased incidence of micronuclei in bone marrow cells from mice that have exposed to 2,4-D TIPA at any dose level.		Unacceptable 008571 Acceptable xxxxxx

TOXCHEM NO. 030053- Dichlorophenoxyacetic acid, 2-Butoxyethyl ester FILE LAST PRINTED: 04/29/92

CITATION	MATERIAL	ACCESSION/ MRID NO.	RESULTS	TOX CAT	COMMENTS/ DOCUMENT#
83-3(a) Developmental Toxicity Study Species: rat Bio/dynamics Inc. 89-3468; 4/25/90	2,4-D butoxyethyl ester tech. 95.6%, lot no. AGR 276426	415271-04	RANGE FINDING STUDY: Levels tested in CD (Sprague-Dawley derived) rats by gavage on days 6-15 of gestation: 0, 73, 145, 218 mg/kg. Maternal NOEL = 73 mg/kg. Maternal LOEL = 145 mg/kg (mortality, decrease body weight gain days 6-9, increased absolute & relative kidney weights, decrease in hemoglobin, hematocrit, and RBC. Develop. Tox. NOEL = not assessed. Develop. LOEL = not assessed.		Supplementary 000186
83-3(a) Developmental Toxicity Study Species: rat Bio/dynamics Inc. 89-3467; 5/9/90	2,4-D butoxyethyl ester, tech. 95.6%; lot # AGR 276426	415271-01	Levels tested in CD (Sprague-Dawley derived) rats by gavage on days 6-15 of gestation: 0, 25, 75 and 185 mg/kg. Maternal NOEL = 75 mg/kg. Maternal LOEL = 185 mg/kg (decr. body weight gain, decr. food consumption, decr. RBC, and incr. reticulocytes). Develop. Tox. NOEL = 25 mg/kg. Develop. LOEL = 75 mg/kg (incompletely ossified interparietal, supraoccipital, squamosal(s), maler(s), and 4th sternebra; presence of 14th rib pairs, short rib(s), rudimentary first lumbar rib(s), and unilateral 14th rib(s). Satisfies 83-3.		Minimum 000186
82-1(a) Feeding-3 month Species: rat Dow Chem; Lake Jackson Res. DECDTXT:K007722015; 01/31/91	2,4-D BEE purity 94.6%	419281-01	Dose levels: 0, 1.5, 22, 145 or 436 mg/kg/day for 13 weeks. No adverse effects at 1.5 or 22 mg/kg/day. At 145 mg/kg/day: decreases in mean body weight, body weight gain & food consumption, alterations in hematology and clinical chemistry parameters changes in thyroid hormone concentrations, and histopathological lesions in the thyroids. At 36 mg/kg/day: body weight loss reduced food consumption, altered hematology and clinical chemistry parameters, changes in various organ weights, changes in thyroid hormone concnetrations, and histopathological changes in the eye, liver, kidneys and thyroids. NOEL = 22 mg/kg/day. LOEL = 145 mg/kg/day.		Guideline 000614
82-2 Dermal-3 week Species: rabbit Dow Chemical Co. K-007722-008; 2/21/90	2,4-D butoxyethyl ester 94.6%	414079-01	Dose levels tested: 0, 50, 150 and 500 mg/kg/day. No treatment-induced dermal irritation. No systemic toxicity at HDT. NOEL => 500 mg/kg for dermal irritation and systemic toxicity.		Minimum 000185
84-2(a) Mutagenic-Ames Species: salmonella Dow Chemical, Texas TXT;K-007722011; 12/12/89	2,4-D butoxyethyl ester (2,4-D BEE)	413882-04 417979-03 420157-01	This study does not satisfy guideline 84-2(a) requirements for mutagenicity testing. A final conclusion can not be reached due to the following reported deficiencies. (1) The spontaneous revertants for the R-factor strain (TA98, TA100) reported in this study are considerably lower than the normal range of revertants for these strains recommended for performing the Ames test. (2) The lack of information as to the test material stability and supporting analytical data to confirm actual test materials concentrations used in the assays. The study may be upgraded upon resolution of reported deficiencies. ~~The additional information/explanation unacceptable. Study can not be upgraded and should be repeated.~~ Evidence given (MRID 420157-01) that incidence of spontaneous histidine revertants for TA98 & TA100 is less in Oxoid L-11 agar than [illegible] this assay that	Unacceptable 007976 000570 Conditionally Acceptable	

009470

PM
PD

TOXCHEM NO. 038053- Dichlorophenoxyacetic acid, 2-Butoxyethyl ester FILE LAST PRINTED: 04/29/92

CITATION	MATERIAL	ACCESSION/ MRID NO.	RESULTS	TOX CAT	COREGRADE/ DOCUMENT#
84-4 Mutagenic-unscheduled DNA sync Species: rat hepatocytes Dow Chem; Lake Jackson Res. TXT:K-087722013; 5/10/90	2,4-D BEE 95.6%	414981-01	Dose levels tested: 5 to 500 ug/mL. Technical problems; excessively high background cytoplasmic and nuclear grain counts in all groups.		Unacceptable 008372 K~~~~~
84-4 Mutagenic-micronucleus assay Species: mice bone marrow Dow Chem; Lake Jackson Res. TXT:K-087722012; 4/24/90	2,4-D BEE 95.6%	414783-01	Dose levels tested: 37.5, 125, and 375 mg/kg-oral. MTD not used; no evidence of toxicity or interaction with target tissue (bone marrow cell) No mortalities occurred (and no symptoms were reported) for animals at the HDT (375 mg/kg).		Unacceptable 008371 K~~~~~

TOXCHEM NO. 000025- Dichlorophenoxyacetic acid, isopropylamine salt FILE LAST PRINTED: 04/29/92

CITATION	MATERIAL	ACCESSION/ MRID NO.	RESULTS	TOX CAT	COREGRADE/ DOCUMENT#
83-3(a) Developmental Toxicity Study Species: rat Bio/dynamics Inc. 89-3466; 3/20/90	2,4-D isopropylamine salt tech. purity 98.2%; lot # AGR 276461	415271-06	RANGE FINDING STUDY: Levels tested in CD (Sprague-Dawley derived) rats by gavage on days 6-15 of gestation: 0, 63, 127 and 190 mg/kg. Maternal NOEL = 127 mg/kg. Maternal LOEL = 190 mg/kg (NOT). (decr. maternal body weight & corrected body weight gain, and increased postimplantation loss). Develop. Tox. NOEL & LOEL not assessed		Supplementary 008186
83-3(a) Developmental Toxicity Study Species: rat Bio/dynamics Inc. 89-3465; 5/7/90	2,4-D isopropylamine salt tech. purity 98.2%; lot AGR 276461	415271-03	Levels tested in CD (Sprague-Dawley derived) rats by gavage days 6-15 of gestation: 0, 22, 65 and 190 mg/kg. Maternal NOEL = 65 mg/kg. Maternal LOEL = 190 mg/kg (NOT) (decreased body weight gain days 6-9, decreased food consumption days 6-11). Develop Tox. NOEL = 65 mg/kg. Develop LOEL = 190 mg/kg (slight increases in skeletal variations).		Minimum 008186
82-1(a) Feeding-3 month Species: rat Dow Chemical Co. M-004725-006; 09/04/91	35.5% 2,4-D acid + 10.8% isopropylamine salt	420214-01	Male and female F-344 rats were fed diets containing 0, 1, 19, 127 or 380 mg/kg/day for 13 weeks. No treatment-related effects at 1 or 19 mg/kg /day. Treatment induced effects at 127 mg/kg/day were decreases in mean body weight gain and food consumption, minor alterations in hematology & clinical chemistry parameters, changes in organ weights, & histopathological changes in kidneys, liver & thyroid. Treatment-related effects at 380 mg/kg/day included decreases in body weight gain & food consumption, alterations in hematology, clinical chemistry & urinalysis, changes in organ weights, gross pathology and histopathological changes in the eyes, liver, kidneys, and thyroid. NOEL = 19 mg/kg/day. LOEL = 27 mg/kg/day based on decreases in body weight & food consumption, alterations in clinical chemistry, increases in relative kidney weights and histopathological lesions in the kidneys, liver and thyroid glands.		Guideline 009405
82-2 Dermal-3 week Species: rabbit Dow Chemical Co. M-004725-004; 2/20/90	2,4-D isopropylamine salt 98.2%	414079-03	Dose levels tested: 0, 50, 125, 350 mg/kg/day. Histopathology revealed inflammation and epidermal hyperplasia at 125 and 350 mg/kg/day groups; no histological lesions were seen at the 50 mg/kg/day. No systemic toxicity was seen. NOEL = 50 mg/kg/day for dermal irritation; 350 mg/kg/d for systemic toxicity. LOEL = 125 mg/kg for dermal irritation.		Minimum 008185
84-4 Mutagenic-unscheduled DNA synt Species: rat hepatocytes Dow Chem; Lake Jackson Res. TXT:M-004725008; 5/3/90	2,4-D IPA 98.2%	414981-03	Dose levels tested: 5-500 ug/mL. technical problems; excessively high background cytoplasmic & nuclear grain counts in all groups.		Unacceptable 008572 Unacceptable ******

009476

TOXCHEM NO. 050025- Dichlorophenoxyacetic acid, isopropylamine salt FILE LAST PRINTED: 04/29/92

CITATION	MATERIAL	ACCESSION/ MRID NO.	RESULTS	TOX CAT	COREGRADE/ DOCUMENT#
84-4 Mutagenic-micronucleus assay Species: mice bone marrow Dow Chem; Lake Jackson Res. TXT:M-004725089; 4/24/90	2,4-D IPA	414783-03	Dose levels tested: 75, 250, or 750 mg/kg-oral. MTD not used; no evidence of toxicity or interaction with target tissue (bone marrow cells). No mortalities and no reporting of any symptoms) at the HDT (750 mg/kg).		Unacceptable 008371 Unacceptable ******
84-2(a) Mutagenic-Ames Species: salmonella Dow Chemical, Texas TXT:K-004725007; ?	2,4-D, isopropylamine salt (2,4-D IPA)	413882-03 417979-02 420157-01	This study does not satisfy guideline requirement. A final conclusion can not be reached due to the following reported deficiencies. (1) The spontaneous revertants for the R-factor (TA98, TA100) reported in this study are considerably lower than the normal range of revertants for these strains recommended for performing the Ames assay. (2) The lack of information as to the test material stability and supporting analytical data to confirm actual test materials concentrations used in the assays. ~~The information/explanation is unacceptable. Study can not be upgraded and should be repeated.~~		Unacceptable 008382 007976 Conditionally Acceptable ******
84-2(a) Mutagenic-Ames Species: salmonella Dow Chemical, Texas TXT;R-004725007; 12/12/89	2,4-D isopropylamine salt (2,4-D IPA)	413882-03	This study does not satisfy guideline 84-2(a) requirements for mutagenicity testing. A final conclusion can not be reached due to the following reported deficiencies. (1) The spontaneous revertants for the R-factor strain (TA98, TA100) reported in this study are considerably lower than the normal range of revertants for these strains recommended for performing the Ames test. (2) The lack of information as to the test material stability and supporting analytical data to confirm actual test materials concentrations used in the assays. The study may be upgraded upon resolution of reported deficiencies. ~~The additional information/explanation unacceptable. Study can not be upgraded and should be repeated.~~		Unacceptable 007976

the studies are the same

Evidence given (MRID 420157-01) that number of spontaneous revertants in TA98 & TA100 is less in Oxoid L-11 agar than in the usually used Bacto-Difco agar. No indication in this assay that the 2,4-D IPA is mutagenic in the

009470

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PESTICIDE ASSESSMENT GUIDELINES

SUBDIVISION F

HAZARD EVALUATION:

HUMAN AND DOMESTIC ANIMALS

Series 84

Mutagenicity

ADDENDUM 9

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FOREWORD

These revised mutagenicity guidelines are intended to replace the set of mutagenicity guidelines originally published in the 1982 Pesticide Assessment Guidelines, Subdivision F, Hazard Evaluation: Human and Domestic Animals, Series 84: Mutagenicity (Office of Pesticide Programs, Washington, DC, publication # EPA-540/9-82-025, pp. 147-151; published again in 1984, publication # EPA-540/9-84-014, pp. 147-151). These newly designated mutagenicity guidelines have been revised in coordination with the upcoming proposed revisions for the Part 158 (Data Requirements for Registration) of the Code of Federal Regulations (40 CFR - Protection of Environment). These revised Subdivision F mutagenicity guidelines have undergone extensive Agency review and public comment as well as review by the Office of Pesticide Programs' Scientific Advisory Panel (SAP). The rationale and decisions behind the revisions as well as responses to the public comments and SAP will be published in a professional journal (manuscript entitled "Considerations in the U.S. Environmental Protection Agency's Testing Approach for Mutagenicity" co-authored by Kerry L. Dearfield, Angela E. Auletta, Michael C. Cimino and Martha M. Moore).

Subdivision F - Series 84

Mutagenicity

Table of Contents

PESTICIDE ASSESSMENT GUIDELINES

SUBDIVISION F

HAZARD EVALUATION:

HUMAN AND DOMESTIC ANIMALS

Series 84: Mutagenicity

84-1 Purpose and General Recommendations for Mutagenicity Testing

(a) When required. As required by 40 CFR (Code of Federal Regulations) Part 158 (Data Requirements for Registration), mutagenicity data shall be submitted to support the registration of each manufacturing-use product and end-use product that meet any of the following criteria:

(1) The use requires a tolerance for the pesticide or exemption from the requirement to obtain a tolerance, or requires the issuance of a food additive regulation; or

(2) The pesticide product is likely to result in significant human exposure; or

(3) The active ingredient(s) or any of its (their) metabolites are structurally-related to a mutagen or carcinogen, or belongs to any chemical class of compounds containing a significant number of mutagens or carcinogens.

(4) See, specifically, 40 CFR Part 158 (section: Formulators' Exemption) and Part 158 (section: Toxicology Data Requirements) to determine whether these data must be submitted. Section II-A of this subdivision contains an additional discussion of the

"Formulators' Exemption" and who must submit the required data as a general rule.

(b) Purpose. For each test substance, required tests are necessary to assess the potential of a test chemical to affect genetic material. Results from such assays may be used as part of a qualitative and/or quantitative risk assessment. The objectives underlying the use of the results from tests for mutagenicity assessment are:

(1) To detect, with appropriate assay methods, the capacity of a chemical to alter genetic material in cells;

(2) To incorporate these findings in

(A) The assessment of heritable genetic alterations of concern to humans (cf. Agency's Guidelines for Mutagenicity Risk Assessment, issued September 24, 1986, 51 FR 34006);

(B) The weight-of-evidence approach for a carcinogenicity classification of a chemical when results from carcinogenicity studies are being considered (cf. Agency's Guidelines for Carcinogen Risk Assessment, issued September 24, 1986, 51 FR 33992). Furthermore, mutagenicity testing information may be helpful in the selection of an appropriate high to low dose risk extrapolation model if the chemical is a demonstrable carcinogen;

(C) The decision to require the performance of a carcinogenicity study if such a study is conditionally required as detailed in Part 158 (section: Toxicology Data Requirements).

(c) Substance to be tested. Testing shall be performed with the technical grade of each active ingredient in the product. Additional testing may require the use of alternative grades of test substance as detailed in Part 158 (section: Requirements for Additional Data).

(d) Standards for metabolic activation. (1) Chemicals are often non-mutagenic unless converted to an active mutagen by metabolic processing. For other chemicals, the reverse may occur, i.e. detoxification. Generally, it is necessary to induce various enzyme activities in order to demonstrate possible mutagenic effects, especially in genetic tests that are of short duration such as assays performed in vitro. Therefore, a metabolic activation system shall be incorporated into any test system that does not provide adequate metabolic capabilities. It is recognized that the species of origin and concentration of liver homogenate, as well as the chemical used as an enzyme inducer, can influence the mutagenic response of in vitro tests.

(2) The test substance shall be tested both in the presence and the absence of mammalian tissue extracts (with appropriate cofactors) which have been demonstrated to convert a wide range of chemical "promutagens" (substances which are mutagenically-inactive in the absence of the tissue extracts) to mutagenically-active substances. Rat liver extracts have had the greatest usage. An example of such an activation system would be cofactor supplemented post-mitochondrial fractions prepared from the livers of rats

treated with enzyme inducers. Aroclor 1254 is typically the most widely used inducing agent in short-term genetic testing; however, if it is shown that other inducing agents are more appropriate for the test chemical, alternative inducing agents may be considered.

(3) Other tissue extracts may be considered in addition to liver extracts when the principal site of metabolism of the test substance is known not to be the liver, or when other tissues, including plant tissue, are known to give positive results with chemicals structurally-related to these chemicals. Hepatocytes may also be considered to provide metabolic activation, either co-cultured with a target cell, or as the primary assay system. As another consideration, the test substance may also be exposed to metabolic processing in intact mammals by a host-mediated system in which the target cells are inserted into host tissues or body cavities. However, before any alternatives to the usual induced-rat liver extracts are used, these should be discussed with the Office of Pesticide Programs (OPP).

(e) Controls. All assays shall be run with concurrent positive and negative controls. Any exceptions to this are found in the testing guidelines for individual assays (see section 84-2, (e)).

(1) Positive controls. Positive control compounds shall be selected to demonstrate both the sensitivity of the test system and, where appropriate, the functioning of the metabolic activation system. For in vitro assays, the positive control compound is usually administered in a solvent that is appropriate to the

properties of the compound. If feasible, the solvent should be the same as the one used for the test chemical. The positive control in many instances is selected in accordance with the performing facility's historical database to allow comparison with previous performances of the assay system. For in vivo assays, where it is feasible, the positive control should be administered in the same vehicle and by the same route as the test chemical. It is recognized that there may be circumstances where this would not be feasible and positive controls administered with a different vehicle and by a different route would be acceptable.

(2) Negative controls. A solvent/vehicle control shall be included with each genetic toxicity test. Although useful information may be obtained from the additional use of a non-solvent negative control for in vitro assays, fully adequate tests need not include a non-solvent negative control.

84-2 Mutagenicity Tests

(a) Tests required. The selection of tests should provide information in accordance with the purposes found in 84-1 (b).

(b) Initial battery. Chemicals that are to be submitted to the Office of Pesticide Programs (OPP) for registration purposes shall be tested in the following test systems in accordance with Part 158 (section: Toxicology Data Requirements):

(1) Salmonella typhimurium reverse mutation assay

(2) Mammalian cells in culture forward gene mutation assay allowing detection of point mutations, large deletions and chromosomal rearrangements, such as:

(A) Mouse lymphoma L5178Y cells, thymidine kinase (tk) gene locus, maximizing assay conditions for small colony expression and detection; or,

(B) Chinese hamster ovary (CHO) or Chinese hamster lung fibroblast (V79) cells, hypoxanthine-guanine phosphoribosyl transferase (hgprt) gene locus, accompanied by an appropriate in vitro test for clastogenicity; or,

(C) Chinese hamster ovary (CHO) cells strain AS52, xanthine-guanine phosphoribosyl transferase (xprt) gene locus.

(3) In vivo cytogenetics (initial consideration usually given to rodent bone marrow) using either:

(A) Metaphase analysis (aberrations); or,

(B) Micronucleus assay

(4) The submitter or the OPP, upon review of the chemical, may assess that other tests may be more appropriate than those in the initial battery. If a substitution and/or modification(s) for a test or tests in the initial battery are suggested, then alternatives to the initial battery shall be discussed with the OPP before the tests are initiated. If tests for endpoints that may be predictive of mutagenicity are performed in addition to the initial battery, the results of such tests shall be submitted to the OPP along with results from the initial battery. Also, as complete a reference list as possible of studies/papers examining the mutagenicity of the test chemical shall be submitted with the submitted mutagenicity tests. Submitters are encouraged to submit all other data relevant to mutagenic activity (e.g. metabolism) as part of their submission.

(c) Confirmatory testing. Testing to confirm results from the initial battery or from other relevant information may be required. This would provide clarification of equivocal results or help resolve discordance among the test results initially submitted to the OPP. Also, additional initial testing may be required to extend the results obtained from the initial battery. One example may be the performance of additional in vivo cytogenetics testing to address such concerns as target tissue/organ or species specificity, differences in metabolism or distribution, as well as structure-activity relationship (SAR) considerations. Another example may be an evaluation for numerical chromosomal alterations.

(d) Decision point. Once testing has been performed in accordance with 84-2 (b) and (c), the OPP will consider the testing results in accordance with the purposes for mutagenicity testing (Section 84-1 (b)).

(1) Decision for no further testing.

The purpose of the initial battery, confirmatory testing and any additional evidence is to generate the appropriate information to discern if the chemical in question poses a mutagenicity hazard or not (i.e. hazard identification). If no mutagenicity hazard is discerned from the body of evidence available to the OPP, there may be no requirement for further testing at this time. If additional future evidence suggests there may be a mutagenic hazard, then the decision for no further testing will be reconsidered.

(2) Decision to continue evaluation for heritable effects.

(A) Further testing to discern potential heritable risk for humans will be considered in accordance with the Agency's Guidelines for Mutagenicity Risk Assessment. This deliberation will consider all mutagenicity test results as well as other considerations, including for example other appropriate toxicity test results (e.g. reproduction, target organ specificity, subchronic and chronic), exposure, SAR aspects, mechanisms, and metabolism. Potential interaction with germ cell target(s) will be particularly examined. Once these deliberations are completed, there may be no requirement for further testing at this time. However, if the weight-of-evidence suggests further testing,

testing that shall be performed may involve cytogenetic testing in spermatogonia and/or spermatocytes of rodents, dominant lethal testing, or testing for other evidence of chemical interaction with germ cells. This would allow an initial determination of potential genetic effects at the target of concern for heritable risk. Further testing to support a quantitative risk assessment will depend upon the results from the available mutagenicity database and other relevant considerations (e.g. exposure of humans and the environment).

(B) When the qualitative evidence using the weight-of-evidence approach as outlined in the Agency's Mutagenicity Risk Assessment Guidelines suggests a potential hazard for heritable mutagenic effects, appropriate tests for quantifying heritable risk shall be performed. Currently, the following are available: the specific locus test (visible or biochemical) and the heritable translocation test, both performed in rodents. A decision to require either or both of these tests would be based upon assessments made up to this point. For example, a chemical with demonstrated mutagenic activity and sufficient evidence of germ cell interaction would be a candidate for such testing. Once results are received upon completion of appropriate tests for quantifying heritable risk, a quantitative risk assessment will be performed in accordance with the Agency's Guidelines for Mutagenicity Risk Assessment.

(3) Decision on evidence to support carcinogenicity classification.

If a chemical has been tested for carcinogenicity in accordance with Part 158 (section: Toxicology Data Requirements), then results of available mutagenicity testing will be used with the carcinogenicity test(s) results as part of the weight-of-evidence approach for classifying the carcinogenicity of the chemical in accordance with the Agency's Guidelines for Carcinogen Risk Assessment.

(4) Decision to require carcinogenicity testing.

When carcinogenicity testing is conditionally required in accordance with Part 158 (section: Toxicology Data Requirements), evidence of chemical mutagenicity may provide the basis to require a carcinogenicity study for that chemical.

(e) Testing guidelines. Guidance for the performance of mutagenicity testing is found in the 40 CFR Part 798 - Health Effects Testing Guidelines, Subpart F - Genetic Toxicity. These guidelines are periodically revised when appropriate to reflect the current state of the science for each test. Where no guideline is given, submitters are advised to discuss with the OPP proposed methods for the chosen test to ensure acceptability of the mutagenicity test and its results. Because of the continual improvements in this field, submitters are encouraged to discuss with the OPP testing battery selection, protocol design and results

of preliminary testing. Testing shall be performed under Good Laboratory Practice (GLP) Standards which are found in the 40 CFR Part 160 (Good Laboratory Practice Standards).

END